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Altering the antigenicity of proteins

(epitope/epitope scanning/synthetic peptides/myohemerythrin/site-directed mutagenesis)

HANNAH ALEXANDER*, STEPHEN ALEXANDER*, ELIZABETH D. GETZOFF†, JOHN A. TAINER†, H. MARIO GEYSEN‡, AND RICHARD A. LERNER†

*Division of Biological Sciences, University of Missouri, Columbia, MO 65211; †Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037; and ‡Chiron Mimotopes Pty Ltd., 11 Duerdin Street, P.O. Box 40, Clayton, Victoria 3168, Australia

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ABSTRACT **To better understand the binding interaction between antigen and antibody we need to distinguish protein residues critical to the binding energy and mechanism from residues merely localized in the interface. By analyzing the binding of monoclonal antibodies to recombinant wild-type and mutant myohemerythrin (MHr) proteins, we were able to test the role of individual critical residues at the highly antigenic site MHr-(79–84), within the context of the folded protein. The results directly show the existence of antigenically critical residues, whose mutations significantly reduce antibody binding to the folded protein, thus verifying peptide-based assignments of these critical residues and demonstrating the ability of buried side chains to influence antigenicity. Taken together, these results (*i*) distinguish the antigenic surface from the solvent-exposed protein surface before binding, (*ii*) support a two-stage interaction mechanism allowing inducible changes in protein antigens by antibody binding, and (*iii*) show that protein antigenicity can be significantly reduced by alteration of single critical residues without destroying biological activity.**

Antigenic epitopes of proteins are composed of continuous or discontinuous portions of the polypeptide chain. The discontinuous epitopes are formed by two or more noncontiguous segments brought into proximity by the folding of the protein. Although much attention has been paid to epitope mapping and characterization of epitopes, there is still no agreement on the precise definition and the make-up of an epitope or on the best ways to identify antigenic epitopes (1–3). It has been suggested that experimental approaches that rely on the linear sequence of the protein to locate and define antigenic determinants would miss many biologically significant sites (4, 5). However, since all discontinuous epitopes contain continuous segments, the study of short peptides can give important information about more complex determinants (6). Indeed, recent studies using peptide-based assays have identified the antigenically important portions of such discontinuous sites (7–13), even to the resolution of single critical amino acids within these sites (14, 15). Studies have also shown that antibodies (Abs) to pathogens, including human immunodeficiency virus (16) and foot-and-mouth-disease virus (17), can recognize or be induced by linear or sequence continuous epitopes.

In our studies of the myohemerythrin (MHr) protein, we have used synthetic peptides to identify a set of amino acid residues that are critical to the immune response, in that their omission or replacement in component peptides results in loss of reactivity with polyclonal and monoclonal anti-MHr Abs (15, 18, 19). However, there has been no direct experimental evidence, for MHr or any other protein antigen (Ag), to verify whether amino acid residues that are identified as antigenically critical by the peptide-based approach are indeed critical within the context of the folded protein. Here, we test directly the correlation between critical side-chain chemistry, as identified by studying synthetic peptides, and the reactivity of monoclonal antibodies (mAbs) with folded recombinant wild-type and mutant MHr proteins.

We have generated a synthetic gene encoding the MHr sequence and have developed an expression system for the recombinant MHr protein (reMHr) (20). A set of reMHr protein analogs, which contain substitutions for MHr-(79–84) epitope residues that were found to be antigenically critical by synthetic peptide-based epitope scanning (18, 19), have been generated by site-specific mutagenesis of the synthetic gene. We show that the substitution of these residues in the protein results in a significant reduction in the reactivity of the mutant protein with mAbs directed to that epitope. Our results confirm and expand the conclusions that were obtained by the peptide-based mapping techniques and support previously proposed models which suggested that the involvement of buried residues occurs by an induced-fit mechanism (6, 15, 18).

MATERIALS AND METHODS

Oligonucleotide Synthesis and Purification. Oligonucleotides for site-directed mutagenesis were synthesized on an Applied Biosystems 380-A DNA synthesizer, using the phosphoramidite method, and purified as described previously (20).

DNA Manipulations. DNA manipulations were performed as described elsewhere (20, 21). Sequencing was performed by using the Pharmacia sequencing kit and the MHr synthetic oligonucleotides as primers.

Expression Labeling and Purification of the reMHr Protein. Labeling and purification have been described elsewhere (20). All purification steps were analyzed by electrophoresis on SDS/12.5% polyacrylamide gels and by ELISA, using antiserum to worm MHr (wMHr) (22). Expression of ^{35}S-labeled reMHr has been described (20).

ELISA. ELISA was performed as described (22), substituting 5% nonfat dry milk (Blotto) for bovine serum albumin as blocking reagent (23).

Competitive ELISA. One picomole of Ag was adsorbed to the wells as described above. Twofold serial dilutions of competing Ag in a final volume of 15 μl were added to each well. Then 15 μl of the indicated Ab dilution was added, and the solutions were mixed and incubated for 90 min at 37°C. The plates were washed, and the ELISA assay was allowed to proceed as described above. Values are presented as percent of reactivity in the absence of competition.

Replacement Studies. The MHr-(79–84) sequence was synthesized on individual plastic pins, where each amino acid

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Abbreviations: Ab, antibody; Ag, antigen; mAb, monoclonal antibody; MHr, myohemerythrin; reMHr, recombinant MHr; wMHr, worm MHr.

was replaced by all other 19 amino acids (representing a total of 120 separate peptides). The reactivity of these peptides with the mAbs was measured by ELISA as described (8), and is expressed as the percent of the reactivity with the parent sequences, after subtracting background from each point.

Absorbance Spectra. Absorbance spectra of wMHr and reMHr were read in a Perkin-Elmer lambda 4B UV/visible spectrophotometer, in the range of 285 to 500 nm. Samples were monitored for an absorbance peak at 328 nm, indicative of the iron center of the properly folded MHr protein (24).

Computer Graphics. The MHr structure (25) was modeled by using AVS software on the Stardent graphics work station.

RESULTS

Mutant Selection. For these studies we have chosen the antigenic site spanning amino acid residues 79–84 of the MHr molecule: Asp-Phe-Leu-Glu-Lys-Ile. This antigenic site represents an α-helix in MHr (Fig. 1), and it participates in a salt bridge and a charged hydrogen bond. This site was strongly recognized by polyclonal anti-MHr Abs produced in rabbits, as well as guinea pigs, chickens, mice, and to some extent sheep (6). There are extensive data on the reactivity of rabbit (8, 22) and mouse (19) Abs with peptides spanning this site. Furthermore, there are mAbs that react with the MHr-(79–84) antigenic site and exhibit preferential sensitivity to substitutions within this epitope (19).

The proposed mutations within the antigenic site are summarized in Table 1. Using computer graphics, we modeled the proposed changes on the three-dimensional structure of the protein to identify their intramolecular interactions. The predictions for reactivity with the mAbs were based on the results of previous experiments, in which the mAbs were allowed to react with hexapeptides representing these sequence variations (19).

Phe-80 and Leu-81 are both hydrophobic, buried residues (Fig. 1). Peptide-based replacement studies found both residues to be essential, as they did not allow even highly

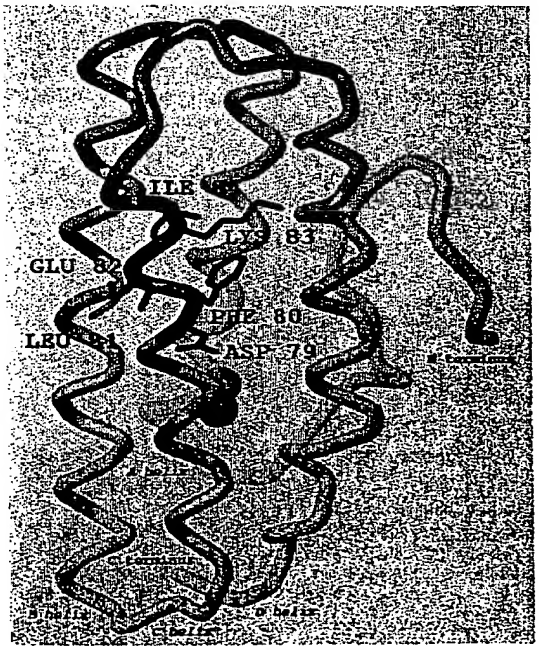

FIG. 1. MHr-(79–84) site in the context of the folded protein. Grey tube shows α-carbon trace. Side chains of residues 79–84 are colored and named. The helices are named A, B, C, and D, and the di-iron center is shown as two orange spheres.

Table 1. Proposed changes in the MHr protein at site 79–84

Residue	Intramolecular interactions*	Change	Predicted reactivity† With mAb A	Predicted reactivity† With mAb M
Phe-80	Buried behind salt bridge	Tyr	−	+
Leu-81	Buried behind salt bridge	Ile	−	+
Lys-83	Exposed, charged	Arg	+	+

*Interactions based upon the wild-type MHr structure.
†Predicted reactivity is from peptide-based scans.

conservative substitutions (19). Conservative substitutions of these residues were made to determine their antigenic role in the context of the folded protein. Lys-83 was chosen for study because it is a hydrophilic residue exposed on the surface of the molecule, and it was found in the replacement studies to be a selectively replaceable residue (15, 18). In this study it was conservatively replaced with Arg. On the basis of our previous peptide-based replacement studies we predicted that this change would not significantly reduce the binding to either of the mAbs.

Site-Directed Mutagenesis and Expression of Mutant Proteins. Mutations were introduced by replacing the fragment encoding the specific residue with newly synthesized oligonucleotides that encoded the modified sequences (20). Whenever possible, additional changes in the DNA sequence, but not the protein sequence, were introduced to eliminate a restriction site for the purpose of screening for plasmids containing the new insertions. Substitution of Tyr for Phe-80

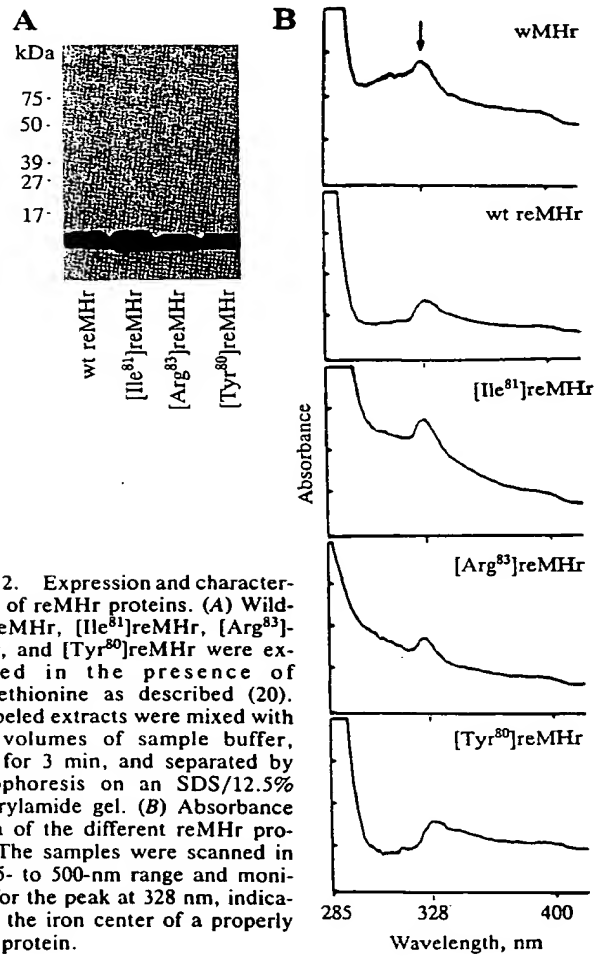

FIG. 2. Expression and characterization of reMHr proteins. (*A*) Wild-type reMHr, [Ile^{81}]reMHr, [Arg^{83}]-reMHr, and [Tyr^{80}]reMHr were expressed in the presence of [^{35}S]methionine as described (20). The labeled extracts were mixed with equal volumes of sample buffer, boiled for 3 min, and separated by electrophoresis on an SDS/12.5% polyacrylamide gel. (*B*) Absorbance spectra of the different reMHr proteins. The samples were scanned in the 285- to 500-nm range and monitored for the peak at 328 nm, indicative of the iron center of a properly folded protein.

was introduced by cloning an *Nsi* I–*Xho* I fragment containing TAC at position 241–243, and a C instead of T at position 234, to eliminate the *Nsi* I site. Leu-81 → Ile was generated by cloning an *Nsi* I–*Xho* I fragment containing A instead of C at position 244. This created a codon for Ile and abolished the *Xho* I site. Arg-83 was generated by cloning an *Nsi* I–*Sal* I fragment containing CGT instead of AAA at 250–252, and a C for T at position 234 to eliminate the *Nsi* I site. All new constructions were verified by direct sequencing, using synthetic oligonucleotides flanking this region as primers.

The wild-type reMHr and the three mutant proteins, containing the substitutions Phe-80 → Tyr, Leu-81 → Ile, and Lys-83 → Arg, were expressed in the T7 polymerase/T7 promoter expression system (26) and purified as described (20). Even though we cannot exclude the possibility of localized conformational changes in the mutants, functional folding was maintained as shown by the correlation between independent measurements of the protein concentration (Lowry assay) and the concentration of the intact di-iron site (A_{328}). Expression of ^{35}S-labeled reMHr protein analogs and their UV/visible absorbance spectra are shown in Fig. 2. The data illustrate that the reMHr protein is expressed at high levels (Fig. 2*A*) and that it folds properly around its iron center (Fig. 2*B*).

Reactivity of Anti-MHr mAbs with Mutant reMHr Molecules. The purified reMHr mutant proteins were analyzed for their reactivity with two different mAbs that were shown by epitope scanning, and by omission studies, to recognize amino acids 79–84 of the MHr molecule. mAb A [$^{(pep)}$mAb A] came from immunization of mice with a synthetic peptide representing the entire C helix of the MHr molecule (residues 68–87), and mAb M [$^{(prot)}$mAb M] came from immunization with the whole wMHr molecule. They both exhibit different fine specificities when analyzed by replacement studies with peptides: $^{(pep)}$mAb A accepted fewer substitutions for most residues within the site, while $^{(prot)}$mAb M was less stringent and exhibited an overall lower affinity for the wMHr molecule. Although not directly applicable to solid-phase assays, the reported K_d values for $^{(pep)}$mAb A (50 nM) and for $^{(prot)}$mAb M (630 nM) define their relative Ab affinities (19).

In Fig. 3 the reactivity of both mAbs with the three reMHr analogs is presented. In each case, the reactivity of the mAbs with reMHr analogs was compared with their reactivity with wild-type reMHr. Controls using anti-wMHr polyclonal antiserum verified that the same amount of reMHr proteins was used in all cases. For assays with 5 pmol of reMHr per well (Fig. 3*A*), the change of Leu-81 to Ile resulted in 10-fold reduction of reactivity of that reMHr species with $^{(pep)}$mAb A, in good agreement with previous studies using peptide-based amino acid replacements (19). In contrast, reactivity with $^{(pep)}$mAb A was affected only marginally by the change of Phe-80 to Tyr, and it was not affected by change of Lys-83 to Arg. $^{(prot)}$mAb M reacted with all species of the reMHr to the same extent, as predicted by the previous peptide-based epitope scanning study.

In experiments using the more stringent concentration of 1 pmol of reMHr per well (Fig. 3*B*) (which is at the K_d for $^{(pep)}$mAb A and far below the K_d for $^{(prot)}$mAb M), the preferential reactivity of $^{(pep)}$mAb A with the three reMHr species was much more pronounced. The substitution Leu-81 → Ile resulted in a 250-fold reduction in the reactivity of that reMHr protein with $^{(pep)}$mAb A as compared with the reactivity with wild-type reMHr. Substitution Phe-80 → Tyr resulted in 10-fold reduction of the reactivity with $^{(pep)}$mAb A. In contrast, substitution Lys-83 → Arg did not affect the reactivity of that reMHr species with $^{(pep)}$mAb A. Under these stringent conditions even $^{(prot)}$mAb M exhibited reduced reactivity with the mutant proteins having Ile at position 81 and Tyr at position 80. Substitution Lys-83 → Arg did not affect the reactivity with $^{(prot)}$mAb M, even under these stringent conditions. These results indicate that, as the stringency of the assay increases, the reactivity of the mAbs with molecules containing mutations in critical residues decreases in comparison with their reactivity with the wild-type MHr.

Competitive ELISA. To determine whether the observed differences in the mAb reactivity with immobilized antigens reflect differences in the affinity of binding, we ran competitive ELISA experiments in which the reactivity of $^{(pep)}$mAb A with the wMHr competed with increasing amounts of various reMHr molecules in solution, and the extent of competition was determined (Fig. 4). Between 10 and 25 pmol of wMHr, wild-type reMHr/wt, and [Arg^{83}]reMHr were needed to reach 50% inhibition. In contrast, 490 pmol of [Tyr^{80}]reMHr and more than 2150 pmol of [Ile^{81}]reMHr were needed to reach 50% inhibition. Thus the substitutions Leu-81 → Ile and Phe-80 → Tyr resulted in a significant loss in binding to $^{(pep)}$mAb A, while change of the noncritical

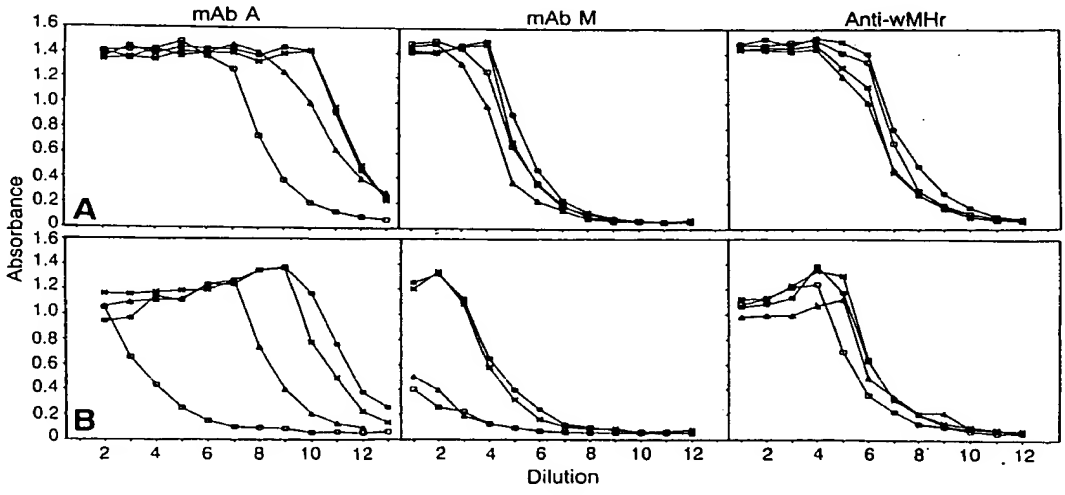

FIG. 3. Reactivity of reMHr with $^{(pep)}$mAb A and $^{(prot)}$mAb M. Amounts of reMHr protein indicated below were adsorbed to wells of microtiter plates and assayed against 2-fold serial dilutions of $^{(pep)}$mAb A (90 pmol per well) and $^{(prot)}$mAb M (100 pmol per well) in ELISA. Absorbance was measured at 405 nm. Since the K_d for $^{(pep)}$mAb A is at least 10-fold higher than that of $^{(prot)}$mAb M, these conditions resulted in a lower overall reactivity of $^{(prot)}$mAb M in the experiments. (*A*) Ag was 5 pmol per well. (*B*) Ag was 1 pmol per well. ■, Wild-type reMHr; □, [Ile^{81}]reMHr; ×, [Arg^{83}]reMHr; ▲, [Tyr^{80}]reMHr.

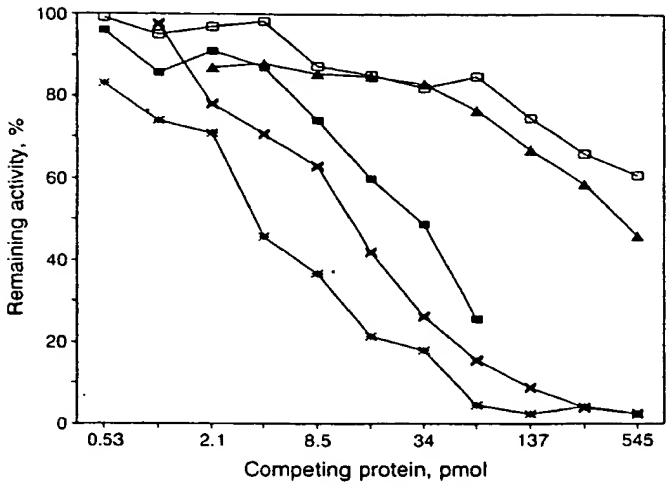

FIG. 4. Competition ELISA. One picomole of wMHr bound to microtiter plates was allowed to react with $^{(pep)}$mAb A, and the reactivity of the bound protein competed with various solution-phase reMHr proteins. Values are presented as percent of the reactivity in the absence of competition, after subtracting background for each point. The concentration of the proteins at the 50% point of the dilution curve was determined. ■, Wild-type reMHr; □, [Ile^{81}]-reMHr; ×, [Arg^{83}]reMHr; ▲, [Tyr^{80}]reMHr; *, wMHr.

residue Lys-83 to Arg did not measurably affect the binding. These data are consistent with the aforementioned solid-phase ELISA experiments (Fig. 3).

Comparison to Peptide-Based Replacement Studies. To follow the effect of the assay conditions on the preferential reactivity of these two mAbs with the different peptide sequences, we repeated the replacement studies (8), using different concentrations of Abs. In Fig. 5 we show the reactivity of $^{(pep)}$mAb A and $^{(prot)}$mAb M with three variant sequences within MHr-(79–84), at four different concentrations of mAbs. The variant sequences were Phe-80 → Tyr (Fig. 5*A*), Leu-81 → Ile (Fig. 5*B*), and Lys-83 → Arg (Fig. 5*C*). The results of these peptide studies exhibit the same trend that was found when assaying the reactivity of the mutant proteins with the mAbs. The changes of Phe-80 to Tyr and Leu-81 to Ile resulted in a considerable reduction of the reactivity of $^{(pep)}$mAb A with these sequences, though the binding was not an all-or-none phenomenon. At high Ab concentrations the reactivity of $^{(pep)}$mAb A with altered sequences is about 50% of its reactivity with the parent sequences, and the effect of the replacements is more pronounced as the concentration of the mAbs decreases. The reactivity of $^{(prot)}$mAb M was not affected by the change of Leu to Ile but was somewhat affected by the change of Phe-80 to Tyr. In contrast, the presence of Arg instead of Lys at position 83 did not affect the reactivity of either mAb with those peptides, demonstrating that Arg is an acceptable substitution at this position.

DISCUSSION

The key question in antigenicity is the nature of the critical residues that contribute most of the binding energy and stabilize the Ag·Ab complex (6). To date, most experiments to dissect the fine specificity of an antigenic determinant have been performed with synthetic peptides, and more recently with phage libraries (27). However, an important remaining issue is whether epitope scanning, using synthetic peptides or phage libraries, reflects the antigenicity, and ultimately the immunogenicity, of the folded protein. The experiments described above test the validity of such results within the context of the folded protein. We have used MHr as a model protein because of the existence of a well-refined x-ray structure, iron sites for monitoring the native conformation spectroscopically (25), and extensive epitope mapping with synthetic peptides (6, 15, 19) and phage libraries (27).

Our data from mutant proteins expand upon earlier work obtained from synthetic peptide experiments and confirm that the residues that were identified as critical to mAb binding in peptide assays are also critical to the binding of the mAbs to the whole protein. In agreement with the peptide studies, our results with the folded protein have found that Phe-80 and Leu-81 are indeed critical to the binding interaction with the $^{(pep)}$mAb A. Under stringent conditions the replacement of these residues results in reduced binding of the mutant proteins to the less restrictive $^{(prot)}$mAb M as well. By contrast, even under the most stringent conditions changing Lys-83 to Arg did not cause a change in the binding of either mAb to this protein species. Thus the peptide-based epitope scanning technique appears to be a rapid and reliable method for determining critical antigenic residues within a folded protein.

Data from peptide replacement nets in the literature, as well as from our previous studies of MHr (15, 19), suggest that substitutions of critical residues can abolish the reactivity of the altered proteins with the mAbs. This study, however, emphasizes that the reactivity pattern of the mAbs with mutant epitopes is not an all-or-none phenomenon. The substitution of critical residues does not eliminate antibody binding, but rather significantly affects the extent of the binding—i.e., the affinity of existing mAbs for the mutant proteins is considerably lower than for the wild-type protein. Under stringent conditions these assays clearly identify the

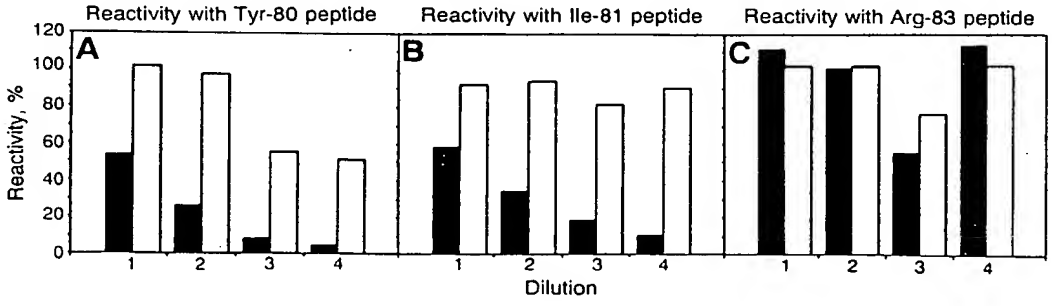

FIG. 5. Replacement assays with $^{(pep)}$mAb A (solid bars) and $^{(prot)}$mAb M (open bars). The mAbs were tested for their reactivity with peptide-coated pins representing the parent, as well as variant sequences of the region MHr-(79–84) (Asp-Phe-Leu-Glu-Lys-Ile) at four different dilutions. $^{(pep)}$mAb A was diluted 25,000-fold, 50,000-fold, 150,000-fold, and 250,000-fold. $^{(prot)}$mAb M was diluted 2500-fold, 5000-fold, 15,000-fold, and 25,000-fold. The dilutions are shown as 1, 2, 3, and 4, respectively. The reactivity with the replacement sequences was calculated as percent of the reactivity with the parent sequence, after subtracting background for each point. (*A*) Phe-80 → Tyr. (*B*) Leu-81 → Ile. (*C*) Lys-83 → Arg.

amino acid residues that are critical to the binding interaction and whose replacement results in a reduced binding. However, under less stringent conditions we can observe reactivity with substituting sequences, in peptide-based experiments as well as in those using the folded protein. In our experiments, the removal of critical residues resulted in a 250-fold reduction in the binding. For an Ab with an affinity for the wild-type Ag in the order of 10^{-7} M to 10^{-8} M this means that the affinity of binding to the mutant protein will be in the range of 10^{-5} M. This should still yield a detectable reactivity under nonstringent conditions.

In proposing the changes for the MHr-(79–84) site, we considered both the nature of the residues and their role in the proposed mechanism of the interaction with the Ab. In site 79–84, the critical and exposed Glu-82 and Lys-83 are involved in interactions stabilizing the structure of the MHr protein. Glu-82 forms a salt bridge with Lys-78, and Lys-83 interacts with Asn-95 through a hydrogen bond. We previously proposed that binding of antibody to Lys-83 will break the hydrogen bond to Asn-95, thus weakening the interaction between the C and D helices. Binding to Glu-82 would break the charge–charge interaction with Lys-78. These concerted changes would allow increased compatibility in the interface and expose the previously buried Phe-80 and Leu-81, making them available to provide a secondary, tighter, binding with the antibody (18). The results obtained in this study support this proposed mechanism. In the folded protein, Lys-83 is assumed to provide a positive charge to the site, and its replaceability with Arg in all studies supports this argument. Leu-81 and Phe-80 were previously found to be critical residues. The fact that Leu-81 and Phe-80 cannot be conservatively replaced with Ile and Tyr, respectively, without decreasing binding argues that these residues are actually binding to the antibodies. The results support the role of these residues in stabilizing the Ag·Ab union after imposing a conformational change on the site. However, their replacement can act both ways, either by removing the binding residue or by imposing local changes such as steric collisions in the epitope.

Whatever the mechanism, these results directly demonstrate the ability of buried side chains to alter antigenic recognition. This has important implications for understanding how pathogens escape the immune system. Aside from direct application of this overall approach for probing the chemical basis of protein antigenicity, applying it to eliminate or alter an immunogenic determinant of a protein may be of significance for vaccine design and replacement therapies where foreign proteins are used.

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